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(54) Title: MODIFIED PF4 COMPOSITIONS AND METHODS OF USE

(57) Abstract

The subject invention pertains to the use of modified PF4 and recombinant PF4 (rPF4) as well as modified analogs (mutants) of PF4, and peptide fragments thereof, to inhibit angiogenesis. The modified PF4, analogs, and certain fragments are shown to have utility for treating angiogenic diseases and for the inhibition of endothelial cell proliferation. Also, the subject invention concerns modifications of PF4 which extend the half-life and facilitate the targeting of the biological activity of PF4 to specific locations.

DESCRIPTION

MODIFIED PF4 COMPOSITIONS AND METHODS OF USE

Cross-Reference to a Related Application

This application is a continuation-in-part of co-pending application Serial No. 07/376,333, filed July 6, 1989.

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Background of the Invention

Angiogenesis, the development of new capillary blood vessels, is an important process in the developing fetus and growing human. However, in healthy adults, angiogenesis occurs significantly only during wound healing and in the menstrual cycle.

10   It is now widely recognized that much of the angiogenic activity occurring in adults is pathological in nature. For example, proliferation of vascular endothelial cells and formation of new capillaries is essential for growth of solid tumors beyond a few cubic millimeters in volume (Folkman et al. [1983] Ciba Found. Symp. 100:132-149). We now understand that developing tumors secrete growth factors which stimulate neighboring 15 endothelial cells to divide and migrate toward the tumor.

In addition to growth of solid tumors, other conditions involving angiogenic dysfunctions include diabetic retinopathy, retrolental fibroplasia, neovascular glaucoma, psoriasis, angiofibromas, immune and non-immune inflammation (including rheumatoid arthritis), capillary proliferation within atherosclerotic plaques, hemangiomas, and Kaposi's 20 Sarcoma have also recently been recognized as diseases possessing characteristics of dysregulated endothelial cell division and capillary growth. These conditions along with growth of solid tumors are collectively referred to as "angiogenic diseases" (Folkman, J., and M. Klagsbrun [1987] Science 235:442-447).

In addition to angiogenic diseases, there are other conditions where endothelial cell 25 proliferation is pathological or, at least, unwanted. For example, endometriosis is characterized by the abnormal proliferation and positioning of certain endothelial cells which normally line the inner wall of the uterus. Control of the angiogenic process could help to prevent or alleviate endometriosis. Also, prevention of endothelial cell growth in the uterus could be a means of birth control.

30   Endothelial cell growth is associated with wound healing. This growth is undesirable during extended surgical proceedings and where excessive scar formation may occur. Therefore, a means of controlling endothelial cell proliferation would help prevent or reduce unwanted scar formation.

The mechanism of angiogenesis and endothelial cell proliferation has not been completely characterized. It has been established that mast cells accumulate at a tumor site before new capillary growth occurs; however, mast cells alone cannot initiate angiogenesis. Heparin, a mast cell product, has been shown to significantly stimulate the capillary endothelial cell migration which is necessary for angiogenesis (Folkman, J. [1984] Angiogenesis: Initiation and Modulation. In Cancer Invasion and Metastasis: Biologic and Therapeutic Aspects. G.L. Nicolson and L. Milas, eds. Raven Press, New York, pp. 201-208).

Several substances are known to have the capability of inhibiting endothelial cell growth in vitro. One of the most extensively studied inhibitors of endothelial cell growth is protamine, which is a protein found only in sperm. Protamine has been shown to inhibit tumor angiogenesis and subsequent tumor growth (Taylor, S. and J. Folkman [1982] Nature 297:307-312). Protamine's anti-angiogenesis activity has been attributed to its well-known capacity to bind heparin (Taylor and Folkman [1982], supra). Clinical experiments with protamine have not been pursued because of the toxicity associated with protamine injection. Protamine, which is usually isolated from salmon sperm, is known to be antigenic in humans, and anaphylactic reactions to this protein have been observed with secondary exposures.

At least two other compounds have been studied in regard to their heparin-binding activity: platelet factor 4 (PF4) and major basic protein. Major basic protein has demonstrated heparin-binding activity but is of little practical utility because of its high toxicity.

Platelet factor 4 is a well-known protein which has been completely sequenced (Deuel, T.F., P.S. Keim, M. Farmer, and R.L. Heinrikson [1977] Proc. Natl. Acad. Sci. USA 74(6):2256-2258). It is a 70-residue secretable platelet protein with a molecular weight of approximately 7.8 Kd. Although there is evidence of heparin binding activity and some indications of anti-angiogenesis activity (Folkman [1984], supra), PF4 has never been shown to have clinical utility.

A compound which has been described as "oncostatin A," and which appears to be the same, or similar to, native PF4, has been implicated as affecting the growth of tumors (U.S. Patent Nos. 4,645,828 and 4,737,580; both issued to Twardzik et al.). However, the effects reported in these patents pertain to slowly growing human cancer cells in immunodeficient mice. The results of these experiments cannot be reliably extrapolated to predict the effect on rapidly growing tumors which are native to the host animal. Furthermore, the experiments reported in these patents in no way predict or disclose any angiostatic properties.

Various peptides from PF4 have been purified and their properties studied. None has been shown to have any role in the inhibition of angiogenesis. It is known that the C-13 peptide of PF4 is chemotactic for neutrophils and monocytes (Deuel, T.F., R.M. Senior, D. Chang, G.L. Griffin, R.L. Heinrikson, and E.T. Kaiser [1981] Proc. Natl. Acad. Sci. USA

78:4585-4587; Osterman, D.G., G.L. Griffin, R.M. Senior, E.T. Kaiser, and T.H. Deuel [1982] Biochem. and Biophys. Res. Comm. 107(1):130-135). It is significant to note that the infiltration of monocytes would be expected to stimulate the proliferation and migration of local endothelial cells by the secretion of angiogenic factors. Thus, peptides of PF4 could be expected to stimulate, rather than inhibit, angiogenesis.

There is a significant and very long-standing need to locate an effective and non-toxic inhibitor of angiogenesis and endothelial cell proliferation. Angiogenesis plays a major role in the initiation and progression of widespread catastrophic illnesses, including cancer. An effective, non-toxic agent which can be administered locally and/or systemically to treat these illnesses would be highly advantageous and has long eluded identification.

Brief Summary of the Invention

The subject invention relates to compositions obtained through chemical modifications of PF4 or recombinant PF4 (rPF4). For example, PF4 can be modified through its free amino groups with fluorescein-isothiocyanate and retain the capability of inhibiting angiogenic activity and endothelial cell proliferation. Similar modifications can be made with PF4 analogs, mutants, or fragments.

A further aspect of the subject invention is the targeting of the biological activity of PF4 to specific locations where that activity is needed. This can be done by conjugating PF4 (or an appropriate fragment, analog, or mutant) to a monoclonal or polyclonal antibody, carrier protein, cell receptor molecule, or other binding protein sequence.

A further aspect of the subject invention is extending the half-life of biologically active PF4 (or appropriate fragments, analogs, or mutants) by conjugating said PF4 to a polymer compound. The polymer may be, for example, a polyamino acid such as polyglutamate, or a polysaccharide such as polyethylene glycol (PEG).

In addition to treating angiogenic disorders and inhibiting endothelial cell proliferation, modified PF4 can also be used to target toxins to specific cell populations. Various other modifications of PF4 and related compounds are described here. These modifications can be made in order to enhance biological activity or otherwise increase the utility of the PF4 compound.

Brief Description of the Drawings

Figure 1 shows the inhibition of angiogenesis resulting from treatment with rPF4 and various related peptides.

Figure 2 compares the amino acid sequence of rPF4 with rPF4-241.

Figure 3 depicts the α-helical configurations of rPF4 and rPF4-241.

Figure 4 compares the inhibition of angiogenesis resulting from treatment with rPF4 and rPF4-241.

Figure 5 shows inhibition of human endothelial cell proliferation by rPF4 and rPF4-241.

Figure 6 compares the inhibition of human umbilical vein endothelial cell proliferation resulting from treatment with rPF4 or rPF4-241.

Figure 7 shows the ability of rPF4 to inhibit tumor growth.

Figure 8 depicts the possible chemical structure of the C-terminal end of FrPF4.

Figure 9 shows inhibition of human endothelial cell proliferation by FrPF4.

Figure 10 shows inhibition of human endothelial cell proliferation by FrPF4-241.

Figure 11 shows inhibition of tumor growth by FrPF4.

Detailed Description of the Invention

The subject invention concerns the discovery that PF4, rPF4, and fragments and analogs of these compounds can be chemically modified to create new compounds with highly desirable characteristics. For example, chemical modification of rPF4 and its fragments has resulted in the identification of compounds which show surprising ability to inhibit angiogenic activity as well as the capability to inhibit endothelial cell proliferation. One specific chemical modification which resulted in altered biological properties involved modification of the free amino groups of rPF4 with fluorescein-isothiocyanate (FITC). The resulting adduct, FrPF4, lacks heparin binding activity because of modification of lysine residues within the heparin binding domain but, surprisingly, retains the ability to inhibit angiogenesis as well as suppress HUVEC proliferation in vitro.

Angiostatic activity is also found in PF4 fragments and mutants which have been modified with the bulky and hydrophobic fluorescein moiety. In addition to their biological activity, the FITC-labeled PF4 sequences are useful for visual detection of PF4 molecules. Furthermore, the ability to modify PF4 and its fragments with large moieties without loss of the relevant biological activity provides a basis for conjugating PF4, its fragments, mutants, or derivatives with toxins, monoclonal antibodies, polyclonal antibodies, fluorophores, cell receptor molecules, non-proteinaceous biological effector molecules, chelators, carrier proteins, polysaccharides, polyamino acids, and other large entities. The conjugation may occur through, for example, modification of functional groups on the protein, such as free amino, carboxyl, sulfhydryl, or guanadinium (arginine) groups of PF4 or a variant of PF4. The modification of these moieties necessary to effect the desired conjugation can be carried out by chemical procedures well known to those skilled in this art.

One of the uses of the compounds described here is in the treatment of angiogenic diseases. As used in this application, the term "angiogenic disease" refers to growth of solid

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tumors, and other conditions involving angiogenic dysfunctions including diabetic retinopathy, retrolental fibroplasia, neovascular glaucoma, psoriasis, angiofibromas, immune and non-immune inflammation (including rheumatoid arthritis), capillary proliferation within atherosclerotic plaques, hemangiomas, and Kaposi's Sarcoma. The subject invention also concerns the use of rPF4 and PF4 fragments, analogs, and mutants for treatment of diseases of dysregulated endothelial cell proliferation.

As used in this application, the term "analog" refers to compounds which are substantially the same as another compound but which may have been modified by, for example, adding additional amino acids or side groups. "Mutants" and "variants" as referred to in this application refer to amino acid sequences which are substantially the same as another sequence but which have amino acid substitutions at certain locations in the amino acid sequence. "Fragments" refer to portions of a longer amino acid sequence.

The subject invention embraces the specific amino acid sequences and other compositions which are specifically exemplified. The subject invention further embraces analogs and mutants of these sequences, as well as fragments of the sequences, and analogs and mutants of the fragments. These analogs, mutants, and fragments are embraced within the subject invention so long as the analog, fragment, or mutant retains substantially the same relevant biological activity as the originally exemplified compound. For example, it is well within the skill of a person trained in this art to make conservative amino acid substitutions. These substitutions are discussed in more detail below. To the extent that these substitutions do not substantially alter the relevant biological activity, then the resulting compounds fall within the scope of the subject invention. The term "relevant biological activity" refers to the activity of interest for a particular application of a compound. For example, several uses of PF4 are discussed below. These uses include inhibition of angiogenesis and endothelial cell proliferation. When PF4 is being used in these ways then "analogs" would refer to compounds where PF4 has been modified (by a conservative amino acid substitution, for example) without substantially altering the compound's ability to inhibit angiogenesis or endothelial cell proliferation. Conservative amino acid substitutions are only one example of the type of modifications which are within the scope of the subject matter of this invention.

The subject invention arises from the unexpected discovery that chemically modified rPF4 inhibits in vivo capillary formation and embryonic neovascularization. It has also been determined that full length recombinant PF4 inhibits growth factor-dependent human endothelial cell proliferation in vitro. Significantly, it has also been determined that the angiogenesis-inhibiting activity of PF4 is retained by synthetic peptides corresponding to sequences of PF4 as small as 10 amino acids in length. In particular, a synthetic peptide of 13 amino acids corresponding to the carboxy terminus of PF4 (C-13) has displayed potent

angiostatic activity. A peptide of 41 amino acids corresponding to the carboxy terminus of PF4 (C-41) has also shown angiostatic activity.

The activity of the C-13 peptide is especially surprising in light of its inability to affect the anticoagulant activity of heparin. The use of the C-13 peptide offers several advantages over whole rPF4 such as reduced dosage (weight basis), reduced likelihood of antigenicity, and greater likelihood of effectiveness in novel dosage forms.

The C-13 peptide of PF4 also retains the ability to prevent Con-A induced immunosuppression in mice, an activity which is unaffected by heparin and probably independent of the ability of the peptide to inhibit angiogenesis.

It is well understood that angiogenesis is required for solid tumors to grow beyond a few cubic millimeters. Thus for the treatment of solid tumors, use of rPF4, or modifications thereof, to cause tumor rejection by inhibiting angiogenesis presents a novel and highly advantageous means of therapy. The fact that the C-13 peptide inhibits angiogenesis without affecting the anticoagulant activity of heparin demonstrates that this small peptide would also have the benefit of not interfering with concurrent anticoagulant therapy. Additionally, small peptides are generally less antigenic than larger proteins, and, thus, the PF4 fragments can be used advantageously for oral and transdermal administration. These types of delivery are particularly useful in the treatment of gastrointestinal capillary proliferation (e.g., Kaposi's Sarcoma) and skin lesions, respectively. Intralesional, as well as systemic, administration of PF4 fragments are also appropriate for treatment of these conditions. Topical or aerosol administration of PF4 fragments is appropriate for skin or pulmonary lesions, respectively (e.g., Kaposi's sarcoma and lung cancer).

An analog of PF4 which exhibits enhanced ability to inhibit angiogenesis has been synthesized. This analog, known as rPF4-241, was created by cassette mutagenesis of a synthetic PF4 gene whereby four lysine residues of the carboxy terminus of PF4 were converted to two Gln-Glu couplets in order to eliminate heparin binding activity while retaining the α-helical secondary structure. If rPF4-241 (or FrPF4-241) is administered intralesionally, it can be applied such that the dosage is between about 1 μg/lesion and about 4 mg/lesion. For systemic administration, the dosage of rPF4-241 (or FrPF4-241) can be between 0.5 mg/kg of body weight and about 100 mg/kg of body weight. Similar and higher dosages can be used for the administration of native sequence rPF4 (or FrPF4) as well as peptide fragments. For example, dosages of rPF4 (or FrPF4) and fragments thereof may be twice that of rPF4-241 (or FrPF4-241) or higher.

The compounds of the subject invention can be combined with a suitable pharmaceutical carrier. For example, FrPF4 or FrPF4-241 can be formulated in physiologically acceptable carriers, such as phosphate buffered saline, distilled water, excipients, or the like, or may be administered neat.

Materials and Methods

Chicken Chorioallantoic Membrane (CAM) Assay. Fertile eggs were incubated in a stationary position for 3 days at 37°C and 70-80% relative humidity. During this time, the embryo rose to the upper surface of the egg contents. At the beginning of the 4th day, the eggs were cracked without inversion and carefully deposited into sterile plastic petri dishes such that the embryo remained on the upper surface. The shell-free eggs were incubated for an additional 72 hours at 37°C, under an atmosphere containing 2.5-3.5% CO_2 after which the growing embryos developed a recognizable CAM. Discs, made by mixing test samples with 1% (w/v) methylcellulose were dried and placed on the CAM between major veins and approximately 0.5 cm from the embryo. Following another 48 hour incubation at 37°C (2.5-3.5% CO_2), the samples were scored for their ability to inhibit angiogenesis. Inhibition appears as an avascular zone surrounding the implant and can often include elbows formed by veins avoiding the disc and a reduced number of capillaries in the region of the implant.

Endothelial Cell Proliferation Assay. Human umbilical vein endothelial cells were cultured in Medium 199 (Gibco) containing 10% (v/v) fetal bovine serum (FBS), 150 mcg/ml endothelial cell growth supplement (ECGS) and 5 units/ml heparin at 37°C and 4-5% CO_2. Every 3-4 days, the cultures were harvested by trypsin treatment, diluted, replated, and grown to confluence. Prior to the start of an experiment, the cells were centrifuged and resuspended in heparin-free media and incubated with the test substance for 3 days under standard culture conditions. At the end of the incubation period, the cells were harvested and counted. Statistical significance between means was determined by a standard Student t-test for unpaired data.

rPF4 Production. Recombinant PF4 was produced in E. coli as an N-terminal fusion protein containing a methionine immediately preceding the PF4 sequence. The insoluble fusion protein was cleaved with cyanogen bromide treatment and purified by heparin agarose affinity chromatography. The isolated protein was buffer exchanged into 20 mM sodium acetate, pH 4.0, and either frozen or lyophilized for storage.

Production of Peptides. Peptides were prepared by standard solid phase synthesis procedures, cleaved from the solid support and deblocked, and purified by reverse phase HPLC.

Following are examples which illustrate procedures, including the best mode, for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1

Chicken eggs, prepared as described above, were treated with discs containing several concentrations of recombinant PF4 or peptides derived from the sequence of PF4. rPF4 and C-terminal peptides as small as 13 amino acids inhibited angiogenesis on the CAM (Figure 1). In each case, the inhibition was dose-dependent and the response approximately equivalent (molar basis) for the inhibitors containing the C-terminal region of PF4. An N-terminal peptide of PF4 (N-29) did not inhibit angiogenesis even at the highest concentration tested, suggesting that all of the anti-angiogenic activity of PF4 is probably associated with the C-terminal portion of the molecule. Since the C-terminus of PF4 is rich in lysine, polylysine was tested in this assay system and found not to cause inhibition at 6.5 nmol dosages.

Example 2

The lysine rich region of PF4 (residues 61-66) is also the domain associated with the binding of heparin by PF4. Heparin is known to play a role in modulating angiogenesis, which can also be affected by protamine, another well characterized heparin-binding protein. To assess the ability of PF4-based synthetic peptides to bind heparin, we assayed the activity of coagulation-cascade enzymes which are inhibited by heparin. Protamine and platelet factor 4 are able to prevent the heparin inhibition of thrombin and Factor Xa at approximately equimolar concentrations. The 41 amino acid C-terminal peptide of PF4 (C-41) prevented heparin inhibition less effectively, but the C-13 peptide was unable to prevent the inhibition of thrombin even at concentrations ten times that of an effective level of rPF4. This unexpected finding suggests that the C-13 peptide inhibits angiogenesis by some method other than heparin binding.

Example 3

Endothelial cell division and growth is tightly controlled and strictly dependent on the presence of growth factors. We evaluated the ability of rPF4 and related peptides to inhibit growth factor-stimulated human endothelial cell proliferation in vitro. rPF4 significantly inhibited endothelial cell growth in a dose-dependent fashion at a concentration as low as 10 mcg/mL. Inhibition was complete at 25 mcg/ml in the heparin-deficient medium employed here.

Example 4

To assess the importance of the heparin binding activity of PF4 in the inhibition of endothelial cell proliferation, cells were incubated in media containing or lacking 5 units/ml heparin. The presence of heparin stimulated proliferation of these cells during the three day

incubation of this experiment. rPF4 significantly inhibited both control (100%) and heparin stimulated (45%) endothelial cell growth (Table 1).

Table 1. Attenuation of rPF4 inhibition of endothelial cell growth by heparin.

Addition	rPF4		% Inhibition[a]
	—	50mcg/ml	
—	14.4 ± 2.5	[b]6.0 ± 0.6	~ 100
5 u/ml heparin	18.9 ± 1.2	[b]14.0 ± 0.4	45

[a]Based on seeding of 8×10^4 cells/well

[b]Significantly different from appropriate control ($p < 0.005$)

Example 5 - Construction of rPF4-241

Cassette mutagenesis of a synthetic PF4 gene was used to convert the four lysine residues at the carboxy terminus of PF4 to two Gln-Glu couplets (see Figure 2). This construction apparently retains the α-helical secondary structure (Figure 3) for this region of the molecule with the concurrent loss of heparin binding activity.

The gene for rPF4-241 was expressed as a fusion protein in E. coli with the same N-terminal amino acid sequences as with the parent rPF4 molecule. The protein was cleaved from the E. coli fusion peptide by CNBr and formic acid and purified to near homogeneity by DEAE-sepharose chromatography. The protein was reactive with polyclonal antibodies to PF4 and was determined to possess the appropriate modifications by amino acid analysis. Significantly, the purified mutant protein lacked heparin-binding activity in the Factor Xa inhibition assay.

The substitutions described here can be made with the peptide fragments as well as with the full length PF4 molecule. For example, C-13-241 has the following sequence:

Pro-Leu-Tyr-Gln-Glu-Ile-Ile-Gln-Glu-Leu-Leu-Glu-Ser

Example 6 - Inhibition of angiogenesis by rPF4-241

Purified rPF4-241 was dried in methylcellulose discs and tested for its ability to inhibit capillary growth in the chicken chorioallantoic membrane (CAM) assay. Even at the lowest concentrations tested (1.25 nmol/disc) rPF4-241 extensively inhibited angiogenesis in the CAM system (Figure 4). This inhibition was even more effective than that caused by equal concentrations of native rPF4 as suggested by larger avascular zones on the membrane. The inhibitory effect of rPF4-241 was not reversed by heparin.

Example 7 - Inhibition of human endothelial cell proliferation by rPF4-241

At concentrations where native rPF4 completely inhibits endothelial cell proliferation, mutant rPF4-241 was at least as effective in inhibiting cell growth (Figure 5). Further tests suggest that rPF4-241 was inhibitory at concentrations as low as 0.5 mcg/mL, a level at which native rPF4 has little or no effect.

In a test of inhibition of human umbilical vein endothelial cell proliferation by native rPF4 and mutant rPF4-241, the rPF4-241 was shown to be much more effective than the native rPF4 at inhibiting the proliferation of these cells. The results of this test are shown in Figure 6.

These results are remarkable in that previous theories of PF4 inhibition of angiogenesis assumed that the PF4 effects were due to heparin binding. We have designed a protein, retaining most of the structural features of native PF4 but lacking detectable heparin binding activity, which is apparently more active than native PF4 in inhibiting angiogenesis in vivo and endothelial cell proliferation in vitro. Additionally, the mutant we have designed would not be expected to interfere with heparin anticoagulant therapy.

Example 8 - Inhibition of *In Vivo* Tumor Growth

Normal C57BL/6J female mice (6-8 weeks old) were inoculated subcutaneously with 5×10^5 log phase cells of a B16-F10 melanoma tumor line. This protocol led to progressive tumor growth resulting in large (300 mm^3) necrotic tumors after approximately 10 days, followed by death of untreated animals usually within three weeks of tumor inoculation.

In an experiment to test the efficacy of rPF4 in preventing in vivo tumor growth and angiogenesis, tumor bearing animals were divided into two groups. One group was injected with 50 µg rPF4 (native sequence) in 100 µl of 50 mM sodium phosphate, pH 6.5, 50 mM sodium chloride directly into the nascent tumor, daily, beginning one day after tumor inoculation. A control group was treated identically with carrier buffer lacking rPF4. Tumor volume was measured at regular intervals with digital calipers by laboratory personnel uninformed of the specific treatment received by each subject animal.

Within seven days of tumor inoculation, control animals possessed obvious three dimensional tumors, while rPF4-treated animals were essentially tumor-free (Figure 7). Continued treatment with rPF4 completely suppressed tumor growth under these conditions where control animal tumors became necrotic and large as seen previously with untreated mice. The same effect was observed when rPF4-241 was used as the inhibitory agent.

This finding supports the proposition that rPF4, as an inhibitor of angiogenesis, will possess clinical usefulness in the management of malignant melanoma and other cancers. Progressive growth of tumors requires new blood vessel formation which, if inhibited, may not

only restrict tumor growth, but stimulate regression of existing vessels, as well as enhance other responses to malignant invasion.

The finding that rPF4 inhibition of in vivo tumor growth was apparent within three days of the initial inoculation (of rPF4) indicates that rPF4 acts to modulate tumor growth by local mechanisms rather than by immunomodulation which would require a longer time course. Additionally, rPF4 did not directly inhibit tumor cell growth in vitro. It appears, therefore, that rPF4 was modulating the host's angiogenic response to the growing tumor.

It has been shown that proteins of identified structure and function may be constructed by changing the amino acid sequence if such changes do not alter the protein secondary structure (Kaiser, E.T., and F.J. Kezdy [1984] Science 223:249-255). Thus, the subject invention includes mutants of the amino acid sequences depicted herein which do not alter the protein secondary structure, or if the structure is altered, the biological activity is retained.

We have conducted extensive research to determine what mutations can be made to the carboxy terminus of the PF4 sequence and still retain biological activity. Table 2 provides a listing of examples of several mutant sequences and their biological activity.

Table 2. Mutant sequences and their biological activity

Designation	Sequence		CAM Activity
		60 70	
rPF4-211 [PF4 AA 1-57] —	Pro Leu Tyr Lys Lys Ile Ile Lys Lys Leu Leu Glu Ser		pos.
rPF4 231 [PF4 AA 1-57] —	Pro Leu Tyr		neg.
rPF4 241 [PF4 AA 1-57] —	Pro Leu Tyr Gln Glu Ile Ile Gln Glu Leu Leu Glu Ser		pos.
rPF4 302 [PF4 AA 1-57] —	Pro Leu Tyr Gln Gln Ile Ile Gln Gln Leu Leu Glu Ser		neg.
rPF4 303 [PF4 AA 1-57] —	Pro Leu Tyr Lys Lys Gln Glu Lys Lys Gln Glu Glu Ser		pos.
rPF4 307 [PF4 AA 1-57] —	Pro Leu Tyr Gln Ile Glu Ile Gln Leu Glu Leu Glu Ser		pos.
rPF4 308 [PF4 AA 1-57] —	Pro Leu Tyr Asn Asp Ile Ile Asn Asp Leu Leu Glu Ser		pos.
rPF4 315 [PF4 AA 1-57] —	Pro Leu Tyr Gly Glu Ile Ile Gly Glu Leu Leu Glu Ser		pos.

Construction of the various mutants was accomplished via cassette mutagenesis of a synthetic gene as described in Example 5 above. This process is well known to any person of ordinary skill in the art. The results of the research demonstrate that a high percentage of the mutants retained angiostatic activity in the CAM assay. Although not every mutant retains this activity, from the teachings herein it is well within the skill of a person trained in this art to make desired mutations and determine whether such activity has been retained.

In particular it should be understood that conservative substitutions of amino acids may be made. For example, amino acids may be placed in the following classes: basic, hydrophobic, acidic, polar, and amide. Substitutions whereby an amino acid of one class is replaced with another amino acid of the same type fall within the scope of the subject

invention so long as the substitution does not materially alter the biological activity of the compound. Table 3 provides a listing of examples of amino acids belonging to each class.

Table 3.

Class of Amino Acid	Example of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Negatively charged	Asp, Glu
Positively Charged	Lys, Arg, His

In some instances, non-conservative substitutions can also be made. For example, lysine may be substituted for with any of the following amino acids: Glu, Gln, Asp, Asn, Met, Ala, Leu, and Ile. The critical factor is that these substitutions must not significantly detract from the biological activity of the rPF4 or the rPF4 fragment.

The following sequence helps provide some additional guidance to a person skilled in this art in making various substitutions. It should be noted that this sequence is meant to be illustrative and not exhaustive, and that there may be other substitutions which eliminate heparin binding and retain angiostatic activity and, thus, are within the scope of the subject invention.

A- Pro Leu Tyr a_{10} a_9 a_8 a_7 a_6 a_5 a_4 a_3 Glu Ser - COOH

wherein A represents all or part of the polypeptide sequence consisting of residues 1 through 57 of PF4; A may be or may not be present;

wherein a_{10} is Lys, Gly, Glu, Gln, Asp, Asn, Met, Ala, Leu, or Ile;

a_9 is Lys, Glu, Gln, Asp, Asn, Met, Ala, Leu, or Ile;

a_8 is Glu, Gln, Met, Ala, Leu, Ile, Val, Pro, Phe, Trp, or Tyr;

a_7 is Glu, Met, Ala, Leu, Ile, Val, Pro, Phe, Trp, or Tyr;

a_6 is Lys, Gly, Glu, Gln, Asp, Asn, Met, Ala, Leu, or Ile;

a_5 is Lys, Glu, Gln, Asp, Asn, Met, Ala, Leu, or Ile;

a_4 is Lys, Glu, Met, Ala, Leu, Ile, Val, Pro, Phe, Trp, or Tyr; and

a_3 is Gln, Met, Ala, Leu, Ile, Val, Pro, Phe, Trp, or Tyr.

wherein, most preferably

a_8 is Ile, Glu, or Gln;

a_7 is Ile or Glu;

a_6 is Leu, Gln, or Glu; and

a_5 is Leu or Glu.

The amino terminus of the proteins of the subject invention can also be modified in a variety of ways while retaining the biological activity fundamental to the subject invention. Most notably, the length of the amino terminus can be modified while retaining angiostatic or endothelial cell inhibitory activity. Our reference here to the "amino terminus" refers to amino acids 1-60 from the amino terminal of PF4 or its variants. As we have shown herein, up to 57 of these amino acids can be removed with a retention of angiostatic activity. The remaining peptide is the biologically active C-13 peptide. We have also shown that the C-41 peptide has biological activity. Thus, various active fragments of PF4 can be readily produced and used according to the subject invention. It would also be readily apparent to the skilled artisan that other modifications of the amino terminus can be made. For example, additional peptides or proteins can be added to that terminus, and various standard chemical derivatives can be made. Such modifications are within the scope of the subject invention so long as biological activity is retained.

It should be emphasized that the critical feature of the subject invention is the provision of polypeptides and polypeptide conjugates which have angiostatic activity or antiproliferative activity but do not bind heparin. As used herein, "angiostatic activity" refers to a level of angiostatic activity which is characteristic of PF4. This level of angiostatic activity is, for example, at least about 75% (and preferably, greater than about 90%) of the angiostatic activity exhibited by PF4 as measured, for example, in the CAM assay. Methods for determining angiostatic activity are described herein and are well known and readily performed by those skilled in this art. As used herein, the term "antiproliferative activity" refers to a level of antiproliferative activity which is characteristic of PF4. This level of antiproliferative activity is at least, for example, about 75% (and preferably, greater than about 90%) of the antiproliferative activity exhibited by PF4 as measured, for example, by the HUVEC assay. Methods for determining antiproliferative activity are described herein and are well known and readily performed by those skilled in this art. As used herein, the term "lack of heparin binding activity" refers to a relative lack of ability to bind heparin under normal physiological conditions compared to PF4. This lack of heparin binding activity is, for example, less than about 25% of PF4's heparin binding activity and, preferably, less than about 10% of PF4's heparin binding activity. The ability to bind heparin can be readily determined by various assays as described herein and as is known by those skilled in this art.

Example 9 - Modification of PF4 and rPF4-241 with Fluorescein-Isothiocyanate (FITC)

Purified rPF4 or rPF4-241 (5 mg in 50 mM Na_2CO_3, pH 9.3, 25 mM NaCl) was treated with 5 mg of fluorescein isothiocyanate in a volume of 5 ml to modify the free amino groups. After incubation for 3 hours at room temperature in the dark, the labeled protein

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(FrPF4 or FrPF4-241).was separated from unbound FITC by gel filtration and dialyzed into 50 mM acetic acid. A possible structure of the C-terminus of FrPF4 is shown in Figure 8.

Example 10 – Inhibition of Angiogenesis by Fluorescein-Isothiocyanate-Conjugated rPF4

FrPF4 was tested for activity in the CAM assay as described above. Although FrPF4 lacked heparin binding activity, it retained full activity as an inhibitor of angiogenesis on the CAM. The results of these assays are shown in Table 5.

Table 5. Activity of FrPF4 in the CAM assay.

Amount per disc (μg)	rPF4	Inhibition (%) FrPF4
0	0	0
10	22	17
25	33	33

Example 11 – Inhibition of Endothelial Cell Proliferation by FrPF4 and FrPF4-241

FrPF4 and FrPF4-241 were tested separately to determine their ability to inhibit endothelial cell proliferation. HUVE cells were tested for their sensitivity to FrPF4 as described above except that [^{3}H]-thymidine was added to the cultures 24 hours after the addition of FrPF4. The cultures were then incubated an additional 6 hours. Cells were harvested, washed, and radioactive thymidine incorporation into DNA was measured.

As shown in Figure 9, FITC-conjugated rPF4 was very effective, even at low dosages, in inhibiting DNA synthesis in human umbilical vein endothelial cells and therefore inhibiting cell proliferation. Similar results were obtained using FrPF4-241. In this case, the inhibition of HUVE cell proliferation with increasing concentrations of rPF4-241 was tested using the Endothelial Cell Proliferation Assay as described above. The results of experiments using FrPF4-241 are shown in Figure 10.

Example 12 – Inhibition of In vivo Tumor Growth by FITC-rPF4

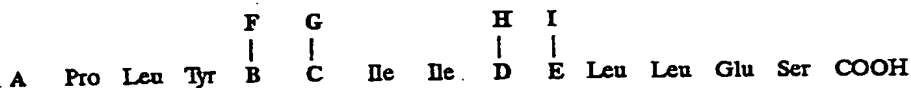B-16 Melanoma tumors were grown in C57BL6/J mice as described previously. Treatment was begun 24 hours following implantation of tumor cells (Day 1) and consisted of 25 μg/day of FrPF4 in 100 μl of sodium acetate buffer, pH 4.0. Control mice were injected with 25 μg/day of FITC labeled cytochrome-C in the same buffer. A statistically significant suppression of tumor growth by FrPF4 was observed by Day 11 (Figure 11).

Example 13 — Delivery of PF4 Activity to Specific Sites

For treatment of certain conditions, it is sometimes advantageous to direct biological activity to a specific location. For example, in order to inhibit solid tumor growth, it may be desirable to send PF4, or an analog with angiostatic properties, directly to the tumor site. This can be accomplished by coupling the PF4 (or analog) to an appropriate antibody, preferably a monoclonal antibody. The monoclonal antibody, which can be produced using techniques that are well-known in the art, will selectively seek out the target site. As the antibody moves to the desired location, it brings with it the PF4. Thus, the PF4 activity can be concentrated at a specific location.

General means of conjugating antibodies to polypeptides such as PF4 are well known to those skilled in the art and are discussed, for example, in U.S. Patent Nos. 4,671,958 (Rodwell et al.) and 4,792,447 (Uhr et al.). The PF4 may also be targeted to specific locations via analogous conjugation with binding proteins (e.g., thrombospondin or fibroblast growth factor), cell receptor molecules (e.g., CD4, lymphocyte function associated antigen-1 [LFA-1], and von Willebrand Factor [vWF]) or the complementary ligands, and non-proteinaceous biological effector molecules (e.g., ICAM-1, tumor associated-antigens, and prostaglandins).

For example, the monoclonal antibody, or other moiety, can be associated with PF4 at one or both pairs of lysine residues located near the carboxy terminus of PF4. By associating the monoclonal antibodies at these residues, the angiostatic activity is retained while heparin binding is eliminated. Also, other amino acid residues may be substituted for the lysine residues before conjugation with appropriate moieties at these and other positions. Therefore, the compounds described here can be represented as follows:

```
                    F    G              H    I
                    |    |              |    |
 A   Pro  Leu  Tyr  B    C   Ile  Ile   D    E   Leu  Leu  Glu  Ser  COOH
where:
```

(a) A represents all or part of the polypeptide sequence consisting of residues 1 through 57 of PF4; A may or may not be present on said hybrid polypeptide;

(b) B, C, D, and E can be any amino acid having a functional group suitable for covalent attachment; and

(c) F, G, H, and I are selected from the group consisting of monoclonal antibodies, polyclonal antibodies, fluorescein-isothiocyanate, fluorophores, toxins, cell receptor molecules, non-proteinaceous biological effector molecules, polyamino acids, polysaccharides, and chelators; at least one of the moieties designated F, G, H, and I must be present on said hybrid polypeptide.

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In the above representation of the compounds described here, the vertical lines represent chemical bonding interactions as do the spaces between the amino acids on the horizontal line. The existence of specifically illustrated moieties associated at B, C, D, and E does not exclude the possibility of conjugation occurring at other residues.

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Example 14 – Conjugation of Carrier Proteins to PF4

It may be desirable to increase the circulating half-life of PF4 to improve its effectiveness as a systemically active angiostatic complex for tumor and angiogenic disease therapy. For example, PF4 can be crosslinked to a large carrier protein, e.g., human serum

10   albumin (HSA) or immunoglobulin, by disuccinimidyl suberate (DSS) through free primary amino groups (i.e., lysine E-amino groups or N-terminal α-amino groups; see Montesano et al. [1982] Biochem. Biophys. Res. Comm. 109:7-13).

Purified rPF4 and HSA (10 mg and 100 mg, respectively) were incubated with 25 mM DSS for 4 hours at room temperature. The reaction was terminated by the addition of Tris

15   buffer, pH 8.0 to a final concentration of 100 mM. The resulting composition was a heterogenous mixture of crosslinked molecules which lacked heparin binding activity, but retained the ability to inhibit HUVEC proliferation. A control sample in which HSA was crosslinked to cytochrome-C did not inhibit HUVEC growth.

20   ### Example 15 – Conjugation of PEG to PF4

An alternative means of extending the half-life of PF4 can be achieved by covalent linkage of PF4 to one or more units of a polymer such as a polyamino acid or a polysaccharide. The polyamino acid may be, for example, polyglutamate, while the polysaccharide may be, for example, polyethylene glycol (PEG).

25   PEG is a water-soluble, non-immunogenic, linear polymer which is available in many well-defined molecular weight ranges (200-20,000 daltons). The dramatic increase in molecular weight of PEG conjugates significantly reduces glomerular filtering of the protein by the kidney. Also, PEG polymers may sterically hinder attack by proteolytic enzymes and immunoglobulin molecules, again adding to the serum life of the protein.

30   Activated esters of PEG are readily coupled to lysine amino groups on a protein. We have successfully employed pH controlled acylation (Katre, N.V., M.J. Knauf, W.J. Laird [1987] Proc. Natl. Acad. Sci. USA 84:1487-1491) to selectively modify the N-terminal amine of peptides in the presence of up to four unprotected lysines. Using the teachings described herein, synthesis of rPF4-PEG conjugates with currently available reagents is straightforward.

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PEG can be covalently attached to rPF4 by free amino groups using a modification of the procedure outlined by Katre et al. (supra). In brief, PEG-glutaryl-N-hydroxysuccinimide (MW-5000, Polysciences, Inc.) is added at a 100-fold molar excess to rPF4 in 0.1 M sodium borate, pH 9.0. The reaction is allowed to proceed at 37°C for 48 hours. Experiments using this protocol have yielded rPF4 with varying degrees of PEG addition. The extent of reaction can be conveniently monitored by SDS-PAGE. Products of the reaction are clearly visible as a series of bands, each differing by 5000 molecular weight and representing species with one or more PEG modifications.

Four lysine residues located near the C-terminus of PF4 have been shown to be essential for binding of PF4 tetramers to heparin. Since these residues are likely sites of PEG modification based on surface accessibility, a subpopulation of rPF4-PEG molecules is synthesized with reduced affinity towards heparin. One possible mechanism for the rapid clearance kinetics observed for PF4 is binding to heparin sulfate present on the surface of endothelial cells. Thus, molecules with reduced heparin binding which retain their anti-angiogenic properties are highly advantageous for systemic efficacy. Coupling reaction products can be fractionated by affinity chromatography on heparin agarose. Major products eluting at different salt concentrations can be analyzed by SDS-PAGE to determine the range of PEG addition in each pool. If desired, each salt fraction can be further separated by other chromatographic techniques (gel filtration or hydrophobic interaction chromatography) to separate molecules with a defined PEG:rPF4 ratio.

If it is desired to retain heparin binding for the rPF4 conjugate, the conjugation process can be carried out in the presence of heparin. The presence of heparin prevents modification of the heparin binding site of PF4 and results in a conjugate which will still bind heparin.

Example 16 – Delivery of Toxin Molecules to PF4 Specific Targets

It is sometimes advantageous to use PF4 as the targeting molecule for directing the activity of a toxic agent to a particular cell type. For example, PF4 can be chemically or genetically crosslinked to the toxin ricin A or the diphtheria toxin.

A fusion protein comprised of PF4 and ricin A can be produced recombinantly in a prokaryotic or eukaryotic host. The resulting purified toxin will have the high specificity for endothelial cells or cells in close proximity to endothelial cells, e.g., tumor cells. Alternatively, PF4 and ricin can be linked with cross linkers. DSS can cross link PF4 and ricin A while retaining both PF4 and ricin A activities.

PF4 can also be covalently linked with a cross linker to photoactivatable molecules, for example, hematoporphyrin derivative (HPD). Water soluble carbodiimides (e.g., EDC) are most useful in linking the acid side chains of HPD to the amino groups of PF4. The resulting

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conjugate will concentrate at sites rich in endothelial cells (such as solid tumors) and can be activated by relatively non-toxic laser or phototherapy focused directly on the tumor site. Activated HPD is known to generate active oxygen species which non-specifically kill nearby cells.

Example 17 – Modification of the Cysteine Residues of rPF4

During preparation, the disulfides of rPF4 are reduced by dithiothreitol (DTT) to free sulfhydryls, but the heparin binding activity is retained. To assess the biological activity of PF4 requires removal of the DTT which, by allowing the disulfide bridges to reform, obscures whether or not they are essential for these activities.

The sulfhydryls of rPF4 were specifically and irreversibly modified by prereduction with DTT followed by treatment with fluorescein maleimide (FM). The reduced and purified rPF4 (5 mg in sodium carbonate buffer, pH 8.5 [SCB]) was treated with 10 mg of FM for 3 hours at room temperature. Residual FM was removed by gel filtration in SCB and then dialyzed against the same buffer. The FM-rPF4 partially retained heparin binding activity. When tested in the CAM and endothelial cell proliferation assays, FM-rPF4 exhibited inhibitory activity indicating that neither free sulfhydryls nor correct disulfide bonds are required for the angiostatic activity of PF4.

This FM-modified rPF4 may possess some utility as an alternative endothelial cell labeling or inhibiting compound but, most importantly, it indicates that the cysteine residues of PF4 are also appropriate targets for conjugating or cross linking PF4 to other molecules for diagnostic or therapeutic applications.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

20

Claims

1. A substantially pure polypeptide conjugate comprising:

(a) a first entity comprising PF4, or a fragment of PF4 comprising at least the 13 amino acids of the carboxy terminus of PF4, wherein said first entity has angiostatic or antiproliferative activity; and

(b) a second entity which is conjugated to said first entity wherein the conjugate retains angiostatic or antiproliferative activity but does not bind heparin.

2. The polypeptide conjugate, according to claim 1, wherein said second entity comprises fluorescein-isothiocyanate or is otherwise the result of treating said first entity with fluorescein-isothiocyanate.

3. The polypeptide conjugate, according to claim 1, wherein said second entity is a fluorophore.

4. The polypeptide conjugate, according to claim 1, wherein said second entity is a toxin.

5. The polypeptide conjugate, according to claim 4, wherein said toxin is the diphtheria toxin or ricin A.

6. The polypeptide conjugate, according to claim 1, wherein said second entity is an antibody.

7. The polypeptide conjugate, according to claim 1, wherein said second entity is a carrier protein.

8. The polypeptide conjugate, according to claim 7, wherein said carrier protein is human serum albumin.

9. The polypeptide conjugate, according to claim 1, wherein said second entity is a chelator.

10. The polypeptide conjugate, according to claim 1, wherein said second entity is a cell receptor molecule or its complementary ligand.

1   11. The polypeptide conjugate, according to claim 1, wherein said second entity is a
2   non-proteinaceous biological effector molecule.

1   12. The polypeptide conjugate, according to claim 1, wherein said second entity is a
2   bulky hydrophobic moiety.

1   13. The polypeptide conjugate, according to claim 1, wherein said second entity is
2   associated with said first entity via modification of one or both pairs of lysine residues located
3   near the carboxy terminus of said first entity.

1   14. The polypeptide conjugate, according to claim 1, wherein said second entity is a
2   photoactivatable molecule.

1   15. The polypeptide conjugate, according to claim 14, wherein said photoactivatable
2   molecule is a hematoporphyrin derivative.

1   16. The polypeptide conjugate, according to claim 1, wherein said second entity is a
2   polyamino acid or polysaccharide.

1   17. The polypeptide conjugate, according to claim 16, wherein said second entity is
2   polyethylene glycol.

1   18. The substantially pure polypeptide conjugate, according to claim 1, comprising
2   (a)   a first entity selected from the group consisting of PF4, C-13, and C-41; and
3   (b)   a second entity which is conjugated to said first entity, wherein said second
4   entity is selected from the group consisting of monoclonal antibodies,
5   polyclonal antibodies, fluorescein-isothiocyanate, fluorophores, toxins, cell
6   receptor molecules, non-proteinaceous biological effector molecules,
7   chelators, polyamino acids, polysaccharides, and carrier proteins.

1   19. A substantially pure polypeptide conjugate comprising:
2   (a)   a first entity which comprises a variant of the following amino acid sequence
3   Gln Glu Ile Ile Gln Glu Leu Leu Glu Ser
4   wherein said first entity has the angiostatic properties or antiproliferative
5   properties characteristic of PF4; and
6   (b)   a second entity conjugated to said first entity.

1   20. The polypeptide conjugate, according to claim 19, wherein said first entity
2 comprises an amino acid sequence selected from the group consisting of:
3 (a)   Lys Lys Gln Glu Lys Lys Gln Gln Glu Ser;
4 (b)   Gln Ile Glu Ile Gln Leu Glu Leu Glu Ser;
5 (c)   Asn Asp Ile Ile Asn Asp Leu Leu Glu Ser; and
6 (d)   Gly Glu Ile Ile Gly Glu Leu Leu Glu Ser.

1   21. A method for the inhibition of endothelial cell proliferation, said method
2 comprising the administration of an effective amount of a composition comprising a
3 polypeptide conjugate wherein said polypeptide conjugate comprises:
4   (a)   a first entity comprising PF4, or a fragment of PF4 comprising at least the 13
5   amino acids of the carboxy terminus of PF4, wherein said first entity has
6   angiostatic or antiproliferative activity; and
7   (b)   a second entity which is conjugated to said first entity wherein the conjugate
8   retains angiostatic or antiproliferative activity but does not bind heparin.

1   22. The method, according to claim 21, wherein said first entity is selected from the
2 group consisting of PF4, C-41, and C-13.

3   23. A method for delivering a toxin to a location with which PF4 interacts, said
4 method comprising the administration of a substantially pure polypeptide conjugate
5 comprising:
6   (a)   a first entity comprising PF4, or a fragment of PF4 comprising at least the 13
7   amino acids of the carboxy terminus of PF4, wherein said first entity has
8   angiostatic or antiproliferative activity; and
9   (b)   a second entity which is conjugated to said first entity wherein the conjugate
10   retains angiostatic or antiproliferative activity but does not bind heparin.

1   24. The method, according to claim 23, wherein said first entity is selected from the
2 group consisting of PF4, C-41, and C-13.

1   25. A method for the inhibition of endothelial cell proliferation, said method
2 comprising the administration of an effective amount of a composition comprising a
3 polypeptide conjugate wherein said polypeptide conjugate comprises:
4   (a)   a first entity comprising a variant of the sequence Gln-Glu-Ile-Ile-Gln-Glu-
5   Leu-Leu-Glu-Ser, wherein said first entity has the angiostatic or
6   antiproliferative activity characteristic of PF4; and

7   (b) _   a second entity which is conjugated to said first entity wherein the conjugate

8   retains angiostatic or antiproliferative activity but does not bind heparin.

1   26. The method, according to claim 25, wherein said first entity comprises an amino

2   acid sequence selected from the group consisting of:

3   (a)   Lys Lys Gln Glu Lys Lys Gln Glu Glu Ser;

4   (b)   Gln Ile Glu Ile Gln Leu Glu Leu Glu Ser;

5   (c)   Asn Asp Ile Ile Asn Asp Leu Leu Glu Ser; and

6   (d)   Gly Glu Ile Ile Gly Glu Leu Leu Glu Ser.

1   27. A method for delivering a toxin to a location with which PF4 interacts, said

2   method comprising the administration of a substantially pure polypeptide conjugate

3   comprising:

4   (a)   a first entity comprising a variant of the sequence Gln-Glu-Ile-Ile-Gln-Glu-

5   Leu-Leu-Glu-Ser, wherein said first entity has the angiostatic or

6   antiproliferative activity characteristic of PF4; and

7   (b)   a second entity which is conjugated to said first entity wherein the conjugate

8   retains angiostatic or antiproliferative activity but does not bind heparin.

1   28. The method, according to claim 27, wherein said first entity comprises an amino

2   acid sequence selected from the group consisting of:

3   (a)   Lys Lys Gln Glu Lys Lys Gln Glu Glu Ser;

4   (b)   Gln Ile Glu Ile Gln Leu Glu Leu Glu Ser;

5   (c)   Asn Asp Ile Ile Asn Asp Leu Leu Glu Ser; and

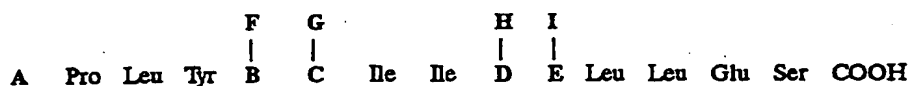6   (d)   Gly Glu Ile Ile Gly Glu Leu Leu Glu Ser.

1   29. A pharmaceutical composition for the treatment of angiogenic diseases, said

2   composition comprising a polypeptide conjugate wherein said polypeptide conjugate comprises

3   (a)   a first entity comprising a variant of the sequence Gln-Glu-Ile-Ile-Gln-Glu-

4   Leu-Leu-Glu-Ser, wherein said first entity has the angiostatic or

5   antiproliferative activity characteristic of PF4;

6   (b)   a second entity which is conjugated to said first entity wherein the conjugate

7   retains angiostatic or antiproliferative activity but does not bind heparin; and

8   (c)   an appropriate pharmaceutical carrier.

1   30.   A pharmaceutical composition for the treatment of angiogenic diseases, said
2   composition comprising a substantially pure polypeptide conjugate, wherein said polypeptide
3   conjugate comprises:

4   (a)   a first entity comprising PF4, or a fragment of PF4 comprising at least the 13
5   amino acids of the carboxy terminus of PF4, wherein said first entity has
6   angiostatic or antiproliferative activity;

7   (b)   a second entity which is conjugated to said first entity wherein the conjugate
8   retains angiostatic or antiproliferative activity but does not bind heparin; and

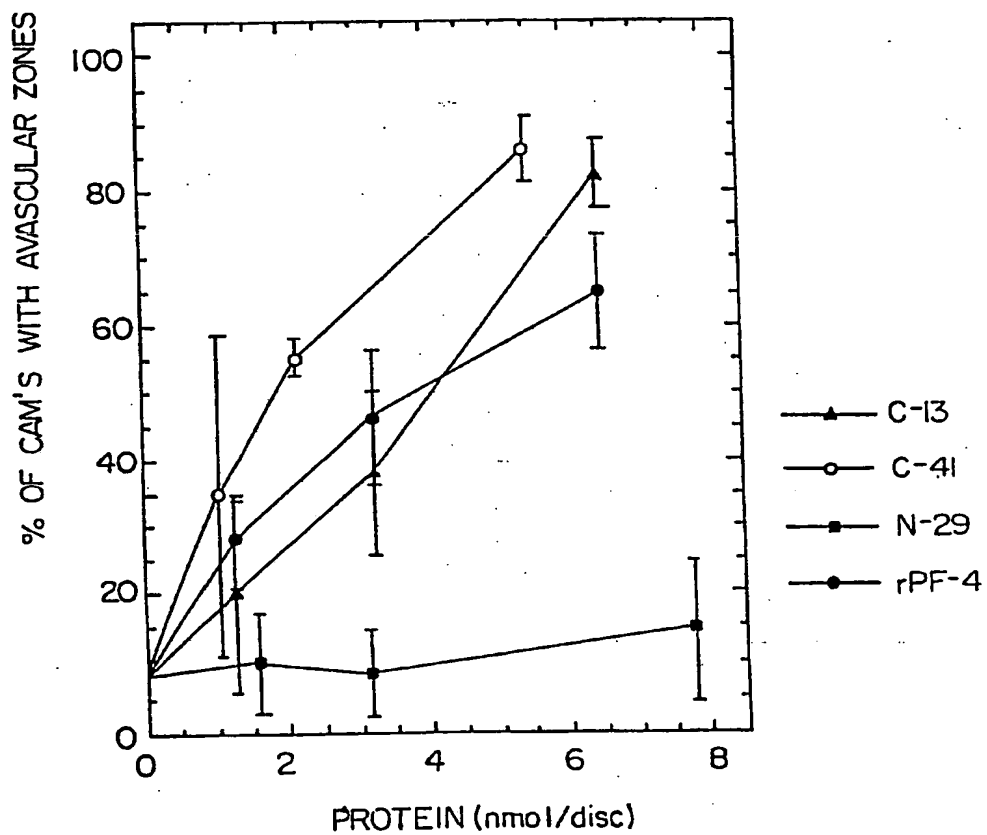9   (c)   an appropriate pharmaceutical carrier.

1   31. A hybrid polypeptide having the following formula:

```
                      F    G          H    I
                      |    |          |    |
   A   Pro  Leu  Tyr  B    C    Ile  Ile  D    E   Leu  Leu  Glu  Ser  COOH
```

where:

6   (a)   A represents all or part of the polypeptide sequence consisting of residues 1
7   through 57 of PF4; A may or may not be present on said hybrid polypeptide;

8   (b)   B, C, D, and E can be any amino acid; and

9   (c)   F, G, H, and I are selected from the group consisting of monoclonal
10   antibodies, polyclonal antibodies, fluorescein-isothiocyanate, fluorophores,
11   toxins, cell receptor molecules, non-proteinaceous biological effector
12   molecules, polyamino acids, polysaccharides and chelators; at least one of the
13   moieties designated F, G, H, and I must be present on said hybrid
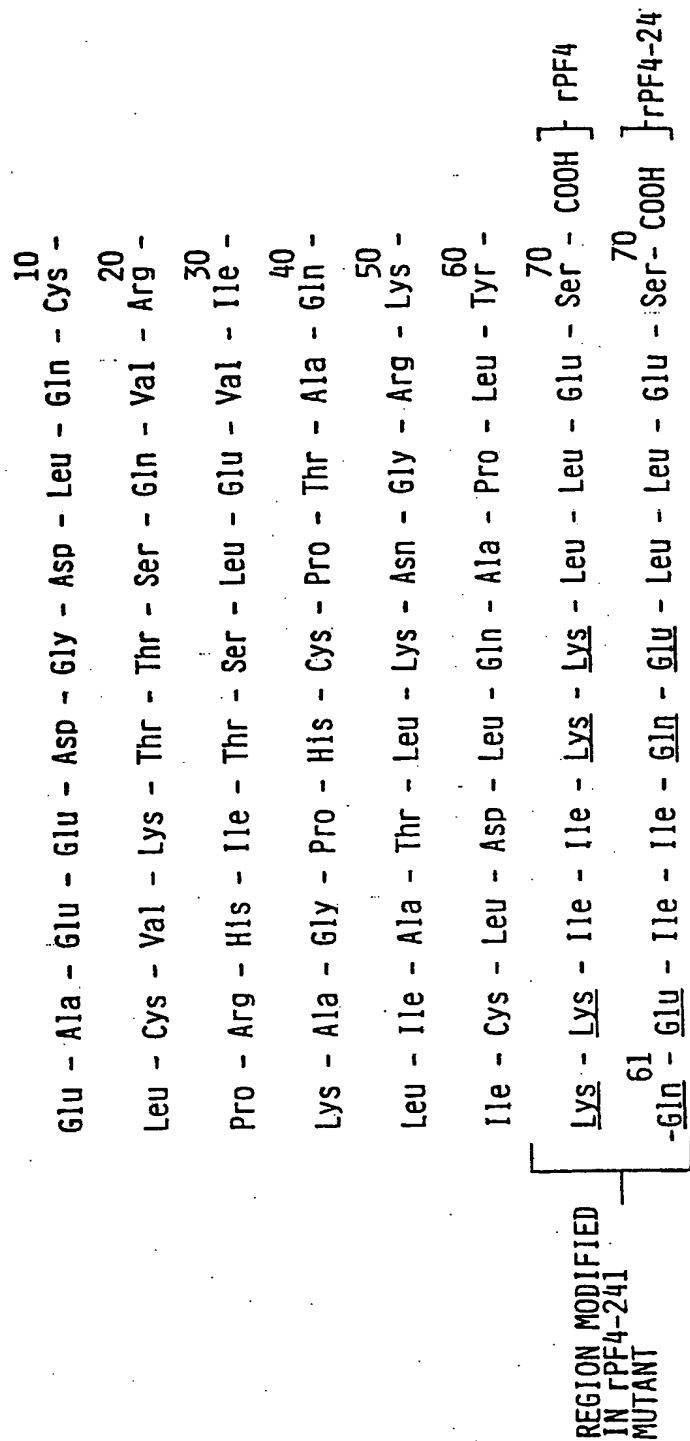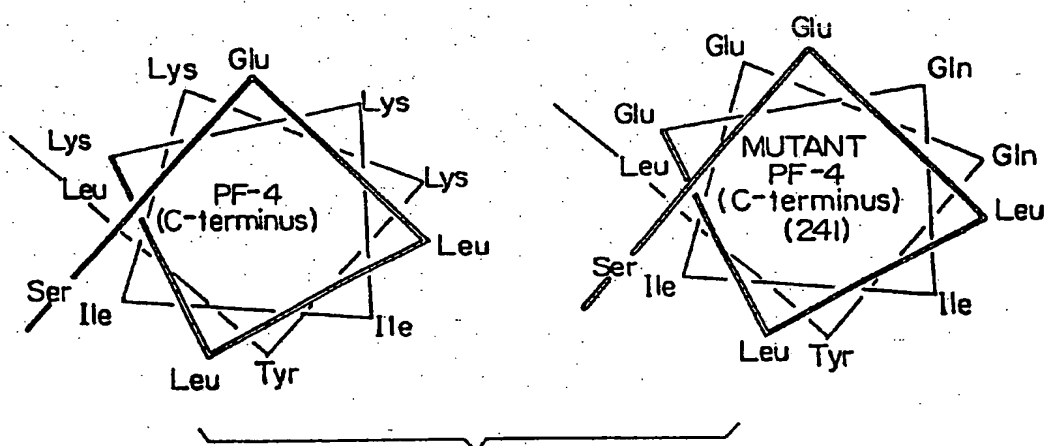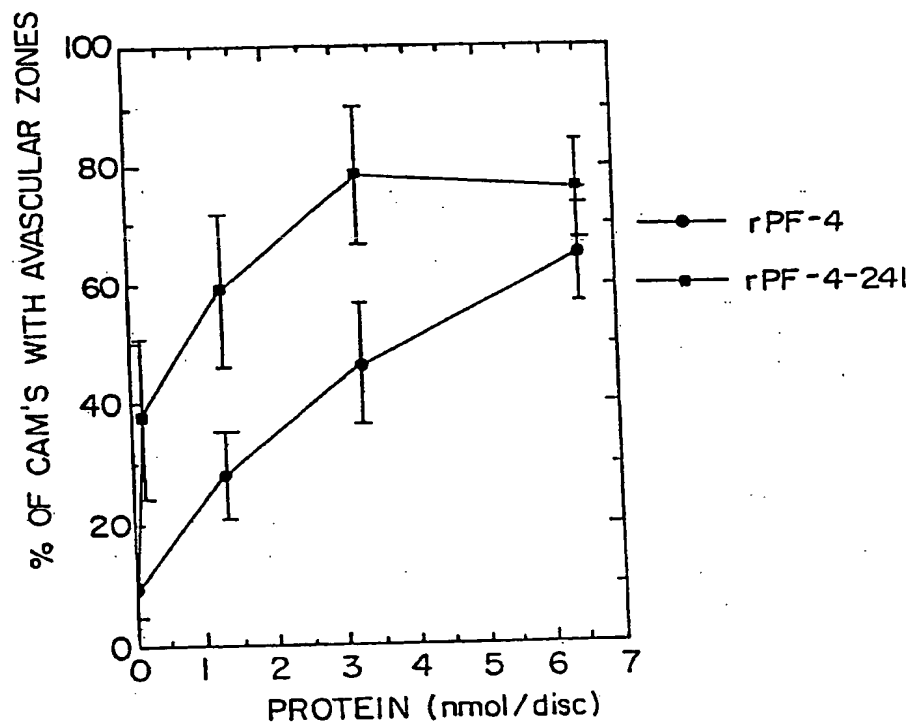14   polypeptide.

//\

FIG. 1

Glu – Ala – Glu – Glu – Asp – Gly – Asp – Leu – Gln – Cys –
                                                        10

Leu – Cys – Val – Lys – Thr – Thr – Ser – Gln – Val – Arg –
                                                        20

Pro – Arg – His – Ile – Thr – Ser – Leu – Glu – Val – Ile –
                                                        30

Lys – Ala – Gly – Pro – His – Cys – Pro – Thr – Ala – Gln –
                                                        40

Leu – Ile – Ala – Thr – Leu – Lys – Asn – Gly – Arg – Lys –
                                                        50

Ile – Cys – Leu – Asp – Leu – Gln – Ala – Pro – Leu – Tyr –
                                                        60

Lys – Lys – Ile – Ile – Lys – Lys – Leu – Leu – Glu – Ser – COOH] rPF4
                                                        70

-Gln – Glu – Ile – Ile – Gln – Glu – Leu – Leu – Glu – Ser– COOH] rPF4-24
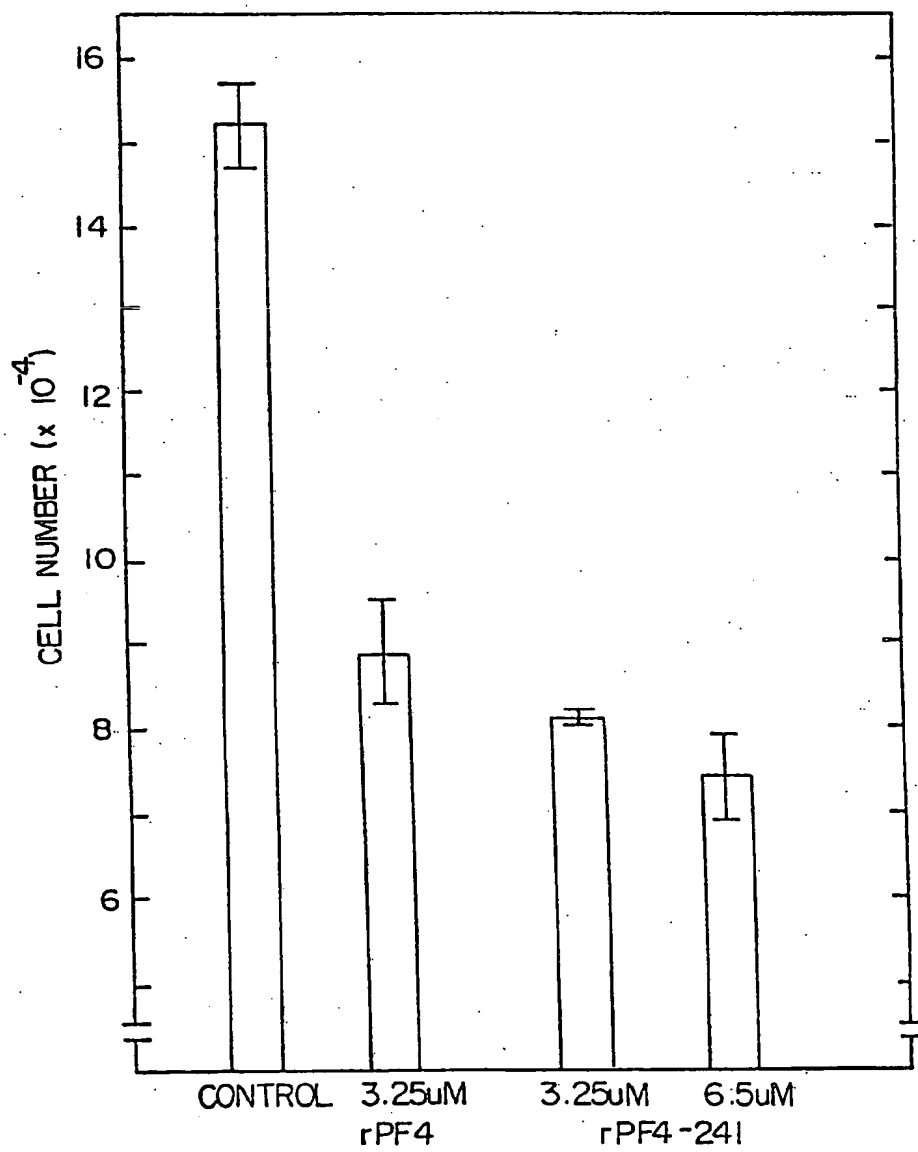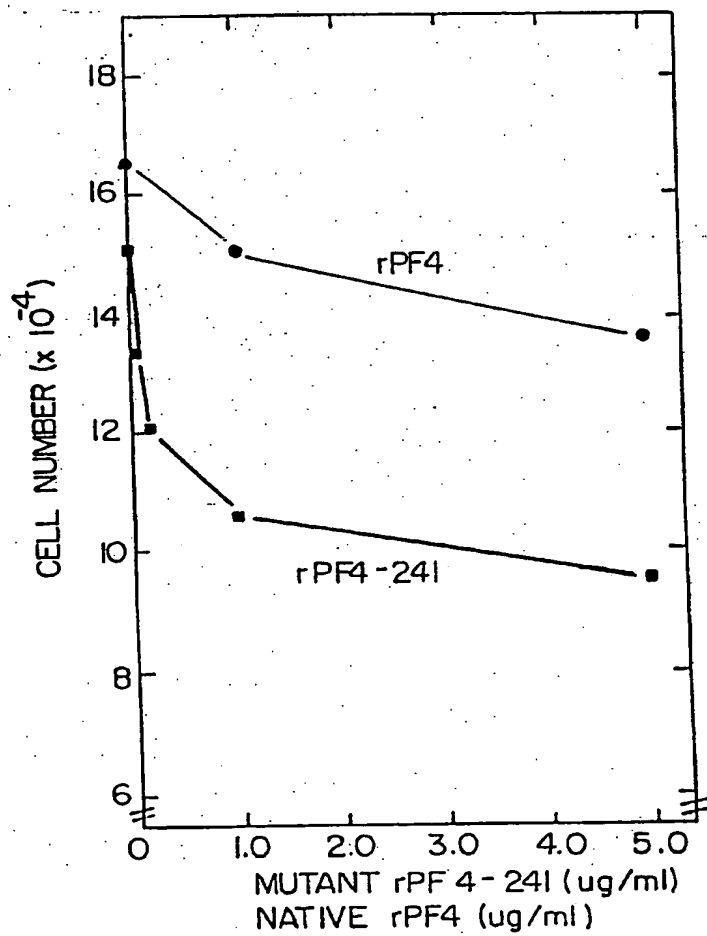 61   70

REGION MODIFIED
IN rPF4-241
MUTANT

FIG.2

FIG. 3

4/11

FIG. 4

FIG. 5

FIG. 6

7/11

FIG. 7

FIG. 8

FIG.9

FITC-rHuPF-4 CONCENTRATION, mcg/ml

FIG. 10

//‖//

FIG. 11

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)⁶			
According to International Patent Classification (IPC) or to both National Classification and IPC			
Int.Cl. 5 C12N15/19; A61K47/48;	C07K7/10; A61K39/395;	C07K7/08; C12P21/02	A61K37/02

II. FIELDS SEARCHED

Minimum Documentation Searched⁷	
Classification System	Classification Symbols
Int.Cl. 5	C07K ; C12N ; C12P ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category°	Citation of Document,¹¹ with indication, where appropriate, of the relevant passages¹²	Relevant to Claim No.¹³
X	EP,A,0 407 122 (REPLIGEN CORPORATION) 9 January 1991	1-15, 18-19, 21-25, 27,29-31
Y	see the whole document	16-17, 20,26,28
Y	EP,A,0 378 364 (REPLIGEN CORPORATION) 18 July 1990 see page 4, line 30 - page 5, line 29 see page 8; example 5	20,26,28
A	see page 9 - page 11; example 9 see claims	1-31

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° Special categories of cited documents :¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20 OCTOBER 1992	02. 11. 92
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	ANDRES S.M.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA vol. 84, no. 5, March 1987, WASHINGTON US pages 1487 - 1491 KATRE, N.V. ET AL. 'Chemical modification of recombinant interleukin 2 by polyethylene glycol increases its potency in the murine Meth A sarcoma model' cited in the application see the whole document	16-17
X	CHEMICAL ABSTRACTS, vol. 112, no. 23, 4 June 1990, Columbus, Ohio, US; abstract no. 213283n, SILBERT, D.I. ET AL. 'Glycosaminoglycans of bovine aorta endothelial cells: identification and localization by use of platelet factor 4-fluorescein probe' page 300 ; see abstract & J.HISTOCHEM.CYTOCHEM., 38(4), 1990, 589-93	1-3,18, 31
A	WO,A,8 806 628 (BRISTOL-MYERS COMPANY) 7 September 1988 see page 25, line 34 - page 26, line 17 see page 38; example 4A see claims	1-31
A	EP,A,0 281 363 (BRISTOL-MYERS COMPANY) 7 September 1988 see page 10, line 12 - line 22; claims 1,15,28-29	1-31
A	EP,A,0 088 695 (CYTOGEN CORPORATION) 14 September 1983 see page 6, line 32 - page 7, line 22 see page 25, line 11 - page 26, line 21 see page 28, line 9 - page 29, line 6 & US,A,4 671 958 cited in the application	1-31

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 21-28 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0407122	09-01-91	US-A-	5112946	12-05-92
		CA-A-	2019086	06-01-91
		JP-A-	3063297	19-03-91
EP-A-0378364	18-07-90	US-A-	5086164	04-02-92
		CA-A-	2007026	10-07-90
WO-A-8806628	07-09-88	AU-B-	623589	21-05-92
		AU-A-	1492488	26-09-88
		EP-A-	0281363	07-09-88
		JP-T-	1503513	30-11-89
		AU-B-	626791	13-08-92
		AU-A-	1559888	10-11-88
		EP-A-	0290948	17-11-88
		EP-A-	0314184	03-05-89
		EP-A-	0372005	13-06-90
		JP-A-	1063395	09-03-89
		JP-T-	4502851	28-05-92
		WO-A-	8903886	05-05-89
EP-A-0281363	07-09-88	AU-B-	623589	21-05-92
		AU-A-	1492488	26-09-88
		JP-T-	1503513	30-11-89
		WO-A-	8806628	07-09-88
		AU-B-	626791	13-08-92
		AU-A-	1559888	10-11-88
		EP-A-	0290948	17-11-88
		EP-A-	0314184	03-05-89
		EP-A-	0372005	13-06-90
		JP-A-	1063395	09-03-89
		JP-T-	4502851	28-05-92
		WO-A-	8903886	05-05-89
EP-A-0088695	14-09-83	US-A-	4671958	09-06-87
		AU-B-	556446	06-11-86
		AU-A-	1199083	15-09-83
		CA-A-	1203164	15-04-86
		DE-A-	3382572	09-07-92
		JP-A-	58222035	23-12-83
		US-A-	5140104	18-08-92

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 20/10/92

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0088695		US-A- 4741900	03-05-88

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82